BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **233,** 265–269 (1997)
ARTICLE NO. RC976365

The *Xanthomonas campestris gumD* Gene Required for Synthesis of Xanthan Gum Is Involved in Normal Pigmentation and Virulence in Causing Black Rot

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Received February 6, 1997

A cloned 4.1-kb *Eco*RI fragment from *Xanthomonas campestris* pv. *campestris* was previously shown to complement the non-mucoid mutant P22 and increase xanthan gum production after being transformed into the wild-type strain Xc17. The gene responsible for these effects was identified, sequenced, and shown to be the *gumD* gene which has previously been proposed to encode glucose transferase activity, an enzyme required for adding the first glucose residue to the isoprenoid glycosyl carrier lipid during xanthan synthesis. A *gumD* mutant, isolated from Xc17 by gene replacement, was shown to possess altered pigment xanthomonadin profiles and exhibit reduced virulence in causing black rot in broccoli. This study appears to be the first to demonstrate that interruption of a gene required for xanthan synthesis can lead to reduced virulence of *X. campestris*.

Xanthan gum is a high molecular weight extracellular heteropolymer produced by the gram-negative bacterium *Xanthomonas campestris* pv. *campestris*. The chemical structure of this substance has been well studied (1, 2). It consists of a cellulosic (1→4)-β-D-glucose backbone with a trisaccharide side chain, possessing the sequence of Mannose-Glucuronic acid-Mannose, attached to alternate glucose residues. A portion of the mannose residues of the side chains are modified by acetylation and pyruvylation. Because of its rheological properties, including high viscosity and pseudoplasticity, and stability in extreme conditions, xanthan gum has been used in food, agriculture and industry as an emulsifying, thickening or suspending agent (3, 4). In addition, xanthan gum has been implicated as the pathogenic substance causing black rot in crucifers (5, 6).

The biosynthesis of xanthan gum involves multiple steps and a multitude of enzymes (7-11). Some of these steps are shared with the synthesis of other polysaccharides, such as lipopolysaccharide (LPS) and peptidoglycan (12). Therefore, defects in any of these steps can cause the failure of xanthan gum synthesis and may affect the synthesis of other polysaccharides. So far, some of the genes required for xanthan gum synthesis have been cloned and the enzyme activities encoded have been detected (13-20). In addition, mutations affecting the mucoid phenotype have been mapped to at least eight different regions of the *X. c.* pv. *campestris* chromosome (21, 22).

Strain P22 was a non-mucoid mutant isolated from Xc11, a less virulent strain, by mutagenesis with nitrous acid (6). Plasmid pP2201 containing a 4.1-kb *Eco*RI fragment cloned from the Xc11 chromosome was able to restore the mucoid phenotype to P22 (23). Furthermore, a 15% increase of xanthan production was observed in Xc17(pP2201) compared to the strongly virulent strain Xc17 (19). In this study, we characterized mutant P22 and found that pigmentation in this mutant was altered. The gene responsible for complementation of P22 was sequenced and found to be the *gumD* gene (GenBank accession # U22511). In addition, the *gumD* mutant derived from Xc17 by gene replacement was found to exhibit reduced virulence in causing black rot.

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Abbreviations: EPS, exopolysaccharide; Km, kanamycin; Km^r, kanamycin-resistance; LPS, lipopolysaccharide; OD, optical density; ORF, open reading frame; Tc^r, tetracycline-resistance.

0006-291X/97 $25.00

MATERIALS AND METHODS

Bacterial strains, media, and cultivation conditions. *X. c.* pv. *campestris* strains Xc11 and Xc17 were wild-type strains (6). P22 was a non-mucoid mutant isolated from Xc11 by mutagenesis with nitrous acid (24). LB and L agar (25) were the general-purpose media for liquid and plate culture, respectively. XOLN (26) was a basal salt medium supplemented with 0.0625% tryptone and 0.0625% yeast extract. Glucose was added into XOLN prior to use at concentrations of 20 and 80 mM, respectively, for measurements of growth and of xanthan production. *Escherichia coli* was grown at 37 °C and *X. c.* pv. *campestris* at 28 °C. Cell growth was measured by reading OD_{550} at intervals of 2 hr.

Measurement of xanthan and protein. The amount of xanthan was measured as previously described using the anthrone method and purified xanthan as the standard (27, 28). Protein was measured by the method of Lowry et al. (29) using the crystalline bovine serum albumin as the standard.

Detection of exocellular enzyme activity. The exocellular enzyme activities of α-amylase, cellulase, pectinase and protease were detected using XOLN plates containing 2% of soluble starch, carboxymethyl cellulose, sodium pectate (Sunkist) or skim milk. Clearing-zones appearing around colonies possessing the respective enzyme activities were readily observed on the plates containing pectate or milk. Starch plates were stored at 4 °C for two days before use to render the plates opaque, which made better contrast for the clearing-zones. This method was better than fumigation with iodine or flooding with iodine solution, since the clearing-zones appear to develop progressively. To examine the clearing-zones produced by cellulase activity, the plates were flooded with 1% copper acetate.

Absorption spectra of xanthomonadin pigments. Extraction of xanthomonadin pigments was carried out as described previously with some modifications (30). Overnight cultures of *X. c.* pv. *campestris* strains grown in LB broth were harvested by centrifugation (10,000 × g, 10 min) in 50 ml polypropyrene centrifuge tubes. After methanol was added at a ratio of 80 ml to 1 g wet weight of cells, the tubes were immersed in a boiling water bath for 5 min. The supernatants were then saved after removing cells by centrifugation. The solutions were concentrated by evaporation under nitrogen gas to give appropriate concentrations for scanning the absorption spectra.

DNA techniques. Restriction endonucleases, Klenow enzyme and T4 DNA ligase were purchased from Promega (Promega Corporation, Madison, Wisconsin) and used according to the supplier's instructions. α-[^{32}P]-dCTP was obtained from Amersham Life Sciences (England, UK). Preparation of plasmid and chromosome, restriction digestion, ligation of DNA, preparation of α-[^{32}P]-labelled probes, DNA hybridization, agarose gel (0.7%) electrophoresis, and transformation of *E. coli* were done by following the procedures described by Sambrook et al. (31). Transformation of *X. c.* pv. *campestris* was performed by electroporation according to the method described by Wang and Tseng (32).

Nucleotide sequence analysis. The DNA fragments to be sequenced were first cloned into M13mp18 and M13mp19. Then nested deletion clones were generated by the non-random deletion method of Dale et al. (33). The nucleotide sequences of both strands were determined by the dideoxy chain termination method of Sanger et al. (34). Nucleotide sequences were analyzed by using Release 6.01 of PC/GENE (IntelliGenetics). Tfasta (35) was used to search in the Genetics Computer Group (GCG) for homologous sequences. Hydrophobicity profiles were plotted by using a hydropathy program with the values of Kyte and Doolittle (36).

Gene replacement. The *gumD* mutant XKSR was derived from Xc17 by gene replacement. The procedure was achieved by the following manner. The 3.0-kb *Sal*I-*Eco*RI fragment from the pP2201 insert was cloned into pUC18, generating pSR3. Then the 1.3-kb Km^r cartridge from pUC4K (37) was inserted into the unique *Sac*I site of the pSR3 insert, forming p3SRK. Then, Xc17 was transformed with p3SRK and scored for Km resistance (50 μg/ml). Since pUC18 is not able to maintain in *X. campestris*, presence of Km resistance in Xc17 could only be resulted from integration of the cartridge into the host chromosome. Integration by double cross-over was verified by Southern hybridization. Using a probe prepared from the 3.0-kb insert of pSR3, a 3.0-kb *Sal*I-*Eco*RI fragment was detected in Xc17, whereas a 4.3-kb *Sal*I-*Eco*RI fragment, containing the 1.3-kb Km cartridge plus the 3.0-kb *Sal*I-*Eco*RI fragment, was detected in the mutant XKSR using the same probe. On the other hand, only the 4.3-kb fragment was detected in XKSR using labelled Km cartridge as the probe.

Pathogenicity test. Pathogenicity was tested as described previously (6), except that the 2-week old potted broccoli seedlings were used.

RESULTS AND DISCUSSION

Characteristics of the Non-Mucoid Mutant Strain P22

On XOLN plates supplemented with glucose, the colonies of P22 were non-mucoid, and were smaller and darker in the yellow color compared to the colonies of the parental Xc11. The size of the P22 colonies ranged from 0.5 to 2 mm which were one third the size of the parental Xc11 colonies. However, in liquid XOLN plus glucose, P22 grew at a rate comparable to that of Xc11, with a doubling time of ca. 150 min. The ability of P22 to utilize sucrose, fructose, galactose, maltose, xylose, or succinate as the carbon sources was similar to that of Xc11.

Both Xc11 and P22 produced the exocellular enzymes α-amylase, protease, cellulase and pectinase. Phage sensitivity tests were carried out by dropping 5 μl of the suspensions of ϕL7 (38) or ϕLf (39) onto a lawn of the host cells. The results showed that P22 remained susceptible to both phages.

X. campestris species produces a mixture of organic solvent-extractable xanthomonadin pigments, which are incorporated into the cell membrane, rendering the cells yellow in color (40). It has been shown that mutations leading to non-mucoid phenotype in *X. oryzae* pv. *oryzae* cause a concomitant alteration in pigmentation (30). Therefore, pigment-containing samples were prepared by methanol extraction and the absorption spectra was scanned. Xc11 showed three absorption peaks at 350, 405 and 445 nm, respectively, similar to the results reported previously for wild-type cells (30, 40). However, samples prepared in parallel from P22 showed a dramatic increase in absorbance at wavelengths from 320 to 400 nm, while the two remaining peaks (405 and 445 nm) remained similar to that of the parental Xc11 (Fig. 1). These data indicate that the ratio of the different xanthomonadins has changed, or that interfering substances were produced at increased amounts as a consequence of the mutation.

Isoprenoid, as the membrane-bound glycosyl carrier, has been shown to participate in the synthesis of

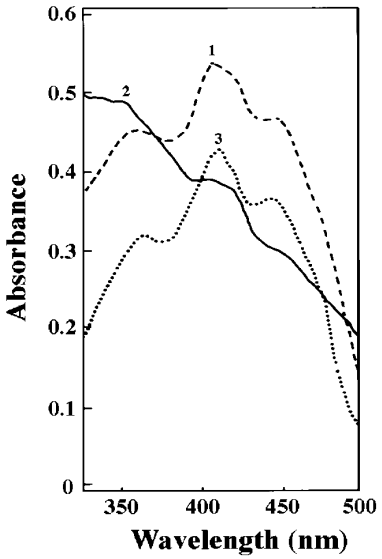

FIG. 1. Absorption spectra of the methanol extract containing the yellow xanthomonadin pigments from *X. c.* pv. *campestris*. Curves 1, 2, and 3 represent samples prepared from strains Xc11, P22, and P22 carrying plasmid pLRB26, respectively.

various polysaccharides including exopolysaccharide (EPS), LPS and peptidoglycan in bacteria (7, 12). Availability of isoprenoid has been proposed to be a possible regulatory step in the synthesis of these polysaccharides (11). Xanthomonadin pigments of *X. campestris* are derivatives of isoprenoid (40). Alteration in pigmentation of P22 with concomitant loss of xanthan synthesis suggests that for pigmentation and xanthan synthesis, a common step for the synthesis and/or source of isoprenoid(s) may be required.

Localization of the DNA Region Complementing Mutant P22

To localize the gene responsible for complementing P22, restriction fragments from the pP2201 insert were subcloned into the broad host range vector pRK415 (41). The clones generated were transformed into P22 and screened for Tc^r (15 μg/ml) and mucoid phenotype. Among five subclones tested, pLRB26 containing the downstream *Bam*HI-*Eco*RI fragment of 2.6 kb was the smallest clone that could restore the mucoid phenotype to P22 (Fig. 2).

Sequence Analysis

The 2.6-kb *Bam*HI-*Eco*RI fragment in pLRB26 (Fig. 2) was subcloned into M13mp18 and M13mp19, and was sequenced. This *Bam*HI-*Eco*RI fragment contains 2,631 nt. Computer search revealed two plausible open reading frames (ORF), both reading rightward. The first ORF, ORF484, starts from the ATG at nt 458 and extends for 484 codons to nt 1,912 where there are two TGA stop codons. The second ORF, ORF II, starts from ATG at position 1,995 and continues for 297 codons to the *Eco*RI site at the end of the fragment without a stop codon, suggesting that ORF II is incomplete. These results suggest ORF484, as the only complete ORF, to be the gene responsible for complementing P22.

Comparison of ORF484 with the sequences in GenBank revealed that the entire region was 98.8% identical to the amino acid sequence of the *gumD* gene product of *X. c.* pv. *campestris* NRRL B-1459 (Accession #U22511), which also contains 484 amino acids. The *gumD* is the third gene of the *gum* gene cluster consisting of 12 genes involved in xanthan synthesis. The GumD has been proposed to possess the glucose transferase activity; mutation in this gene results in the failure to add glucose, which is the first residue in the synthesis of xanthan gum subunit, to the isoprenoid carrier lipid (42, 43). Comparing to the GumD in NRRL B-1459, the ORF484 product had six amino acids changed in: $Val_{93} \rightarrow Met$, $Val_{124} \rightarrow Ile$, $Met_{185} \rightarrow Leu$, $Ala_{198} \rightarrow Thr$, $Glu_{199} \rightarrow Asp$ and, $Val_{259} \rightarrow Asp$. Sequence comparison also revealed that ORFII is the homolog of *gumE* of NRRL B-1459 which is the fourth gene of the *gum* gene cluster (44; Accession # U22511). Recently, transcriptional start site for *gum* gene cluster has been determined 158 nt upstream to the *gumB* gene (44).

In addition, the C-terminal 195 amino acids of ORF484 product shows 44% identity to PssA (200 amino acids) of *Rhizobium leguminosarum* bv. *phaseoli* required for normal synthesis of EPS and nodulation in peas, as well as an overproduction of EPS when present at a high copy number (45). The PssA contains many hydrophobic amino acids (49%), especially at the N-terminus, and has been shown to be membrane-associated (46). The C-terminal 195-amino acids of the ORF484 product has 45% hydrophobic amino acids and shows similar hydrophobicity profiles to that of PssA (Fig. 3). These similarities suggest that they may have similar functions and GumD may also be a membrane-associated protein.

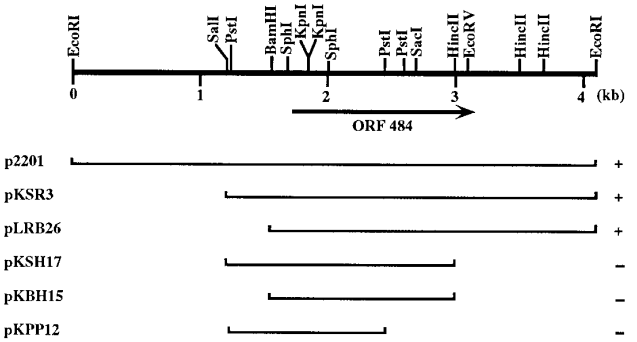

FIG. 2. Restriction map of pP2201, position of *gumD* gene, and the restriction fragments from the pP2201 insert cloned into pRK415. Positive and negative results of complementation experiments are indicated by "+" and "–," respectively.

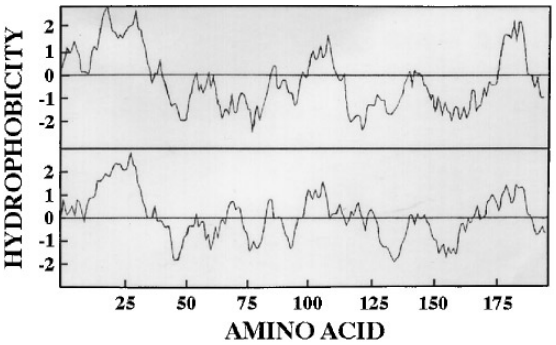

FIG. 3. Comparison of protein hydrophobicity profiles of the C-terminal 195 amino acids of GumD of *X. c.* pv. *campestris* strain 11 and the PssA of *Rhizobium leguminosarum* bv. *phaseoli*. The profiles were plotted according to a hydropathy program with the values provided by Kyte and Doolittle (36).

Isolation of gumD Mutant by Gene Replacement

EPS and LPS produced by plant pathogenic bacteria have been correlated to pathogenicity (21, 47, 48). In *X. c.* pv. *campestris*, controversial results have been reported concerning the role played by xanthan gum in causing black rot in crucifers (5, 6, 49). We have previously shown that, after repeated subculturing, cells of Xc11A produced less xanthan and lost virulence (6). Since P22 had also been subcultured for many generations, it was difficult to judge whether it was suitable for pathogenicity tests. Therefore, in order to test the effects of *gumD* mutation on pathogenicity, a *gumD* mutant was isolated from the strongly virulent strain Xc17 by gene replacement. This mutant, designated XKSR, carried a Km cartridge inserted in the chromosome at the unique *Sac*I site within the *gumD* coding region.

Colonies of XKSR were non-mucoid, and smaller in size, and darker in the yellow color compared to the colonies of Xc17. Similar to P22, XKSR retained all the other wild-type characteristics tested. Only residual amounts of xanthan gum were produced by XKSR. The amounts measured for Xc17, XKSR(pP2201) and XKSR were 3,460, 1,160 and 160 μg/ml, respectively. It is noteworthy that the ability of XKSR to synthesize xanthan was only partially restored by pP2201 (ca. 33.5% of the Xc17), different from the case in P22 where the xanthan synthesis was completely restored by pP2201. These results indicate that polar effects might have resulted from the insertion of a Km cartridge into the *gumD* gene.

The gumD Mutant Exhibited Reduced Virulence

To test for pathogenicity, the cells of Xc17, XKSR(pP2201) and XKSR were inoculated onto the young leaves of broccoli seedlings. After 3 days, symptoms caused by Xc17 were observed. It was a typical chlorosis, resulting in a yellowing and then browning of the area around the cut. The symptoms caused by XKSR(pP2201) appeared at 6 days postinoculation, whereas the symptoms caused by XKSR were only visible about 6 more days later. In other words, XKSR exhibited much reduced virulence compared to the wild-type Xc17 (Fig. 4).

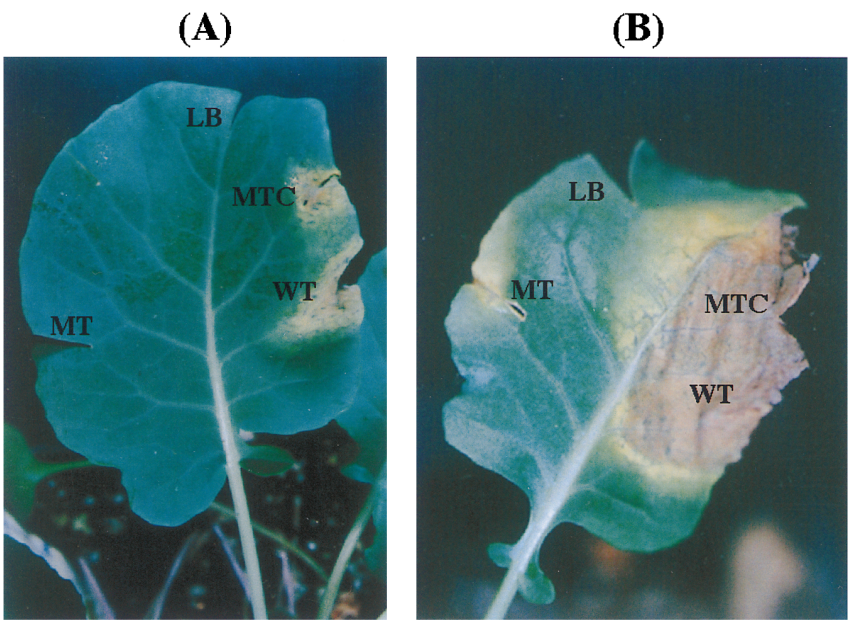

FIG. 4. Chlorosis symptoms in broccoli leaves caused by Xc17 (WT), *gumD* mutant XKSR containing pP2201 (MTC), and XKSR (MT). LB medium was used as the control (LB). Shown in (A) and (B) are the symptoms observed at 6 and 15 days, respectively, after inoculation.

Although the ability of xanthan gum synthesis has been implicated to be correlated with pathogenicity of *X. c.* pv. *campestris*, this study appears to be the first to demonstrate the correlation by mutation of a specific gene involved in the synthesis of xanthan gum.

ACKNOWLEDGMENTS

This study was supported by Grants NSC 84-2311-B-005-022 and NSC 85-2311-B-005-018 B18 from the National Science Council, Republic of China. We thank M. Arab for reading the manuscript.

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